Reference: *Biol. Bull* **185**: 215–224. (October, 1993)

Histochemical Studies of Jelly Coat of *Marthasterias glacialis* (Echinodermata, Asteroidea) Oocytes

MÁRIO SOUSA[1,*], RUI PINTO[3], PEDRO MORADAS-FERREIRA[2], AND CARLOS AZEVEDO[1]

Laboratories of [1]Cell Biology and [2]Biochemistry of the Institute of Biomedical Sciences, University of Oporto, and [3]the Laboratory of Enzymology of the Institute of Medical Genetics, 4000 Porto, Portugal

Abstract. Histochemical studies revealed the presence of two major polysaccharides in the oocyte jelly coat (JC) of *Marthasterias glacialis:* a fibrillar component that contains carboxylic and sulfated groups and a loose component composed of neutral or weakly acidic polysaccharides. When isolated JC was submitted to cellulose acetate electrophoresis (CAE) and then stained with alcian blue, three bands appeared, of which one remained at the origin and two migrated toward the anode. Glycosaminoglycan-like molecules isolated from JC were separated by CAE into three main moving bands, two that present an R_f similar to that of the intermediary moving band of total JC and one that has an R_f similar to that of the faster moving band of total JC. These bands also have critical electrolyte points similar to those of total JC. Chondroitinase ABC mainly attacked the faster moving band, whereas protease and hyaluronidase seemed to digest all bands. These results and the R_fs of isolated and standard glycosaminoglycans after mono- and bidimensional CAE suggest that the glycosaminoglycan-like molecules bear some resemblance to chondroitan sulfate, heparan sulfate, and hyaluronic acid.

Introduction

Complex carbohydrates have been implicated in sperm-egg recognition, binding, and activation in both vertebrates and invertebrates (Miller and Ax, 1990).

The macromolecular structures of the jelly coat (JC) of the echinoderm oocyte have not yet been morphologically differentiated, despite the importance of this information to the understanding of sperm-egg interaction. In the sea urchin, the oocyte JC was demonstrated to contain a sialoprotein and a fucose sulfate polysaccharide that induces the acrosomal reaction of the sperm (SeGall and Lennarz, 1979). Histochemical studies have also found two major polysaccharides in the JC, neutral and acidic (Jondeung and Czihak, 1982), thus confirming the biochemical results. In the starfishes *Asterias amurensis* and *Asterina pectinifera*, the oocyte JC was also demonstrated to contain two major polysaccharides, a large sulfated glycoprotein that induces the acrosomal reaction and a high-mannose glycoprotein (for a review see Hoshi *et al.*, 1990a, b), but no morphological studies have been made to confirm these biochemical findings.

We characterized the oocyte JC of the starfish *Marthasterias glacialis* by histochemical and electrophoretical methods. Our results show that the JC can be morphologically differentiated into two major polysaccharides that probably contain glycosaminoglycan-like materials.

Materials and Methods

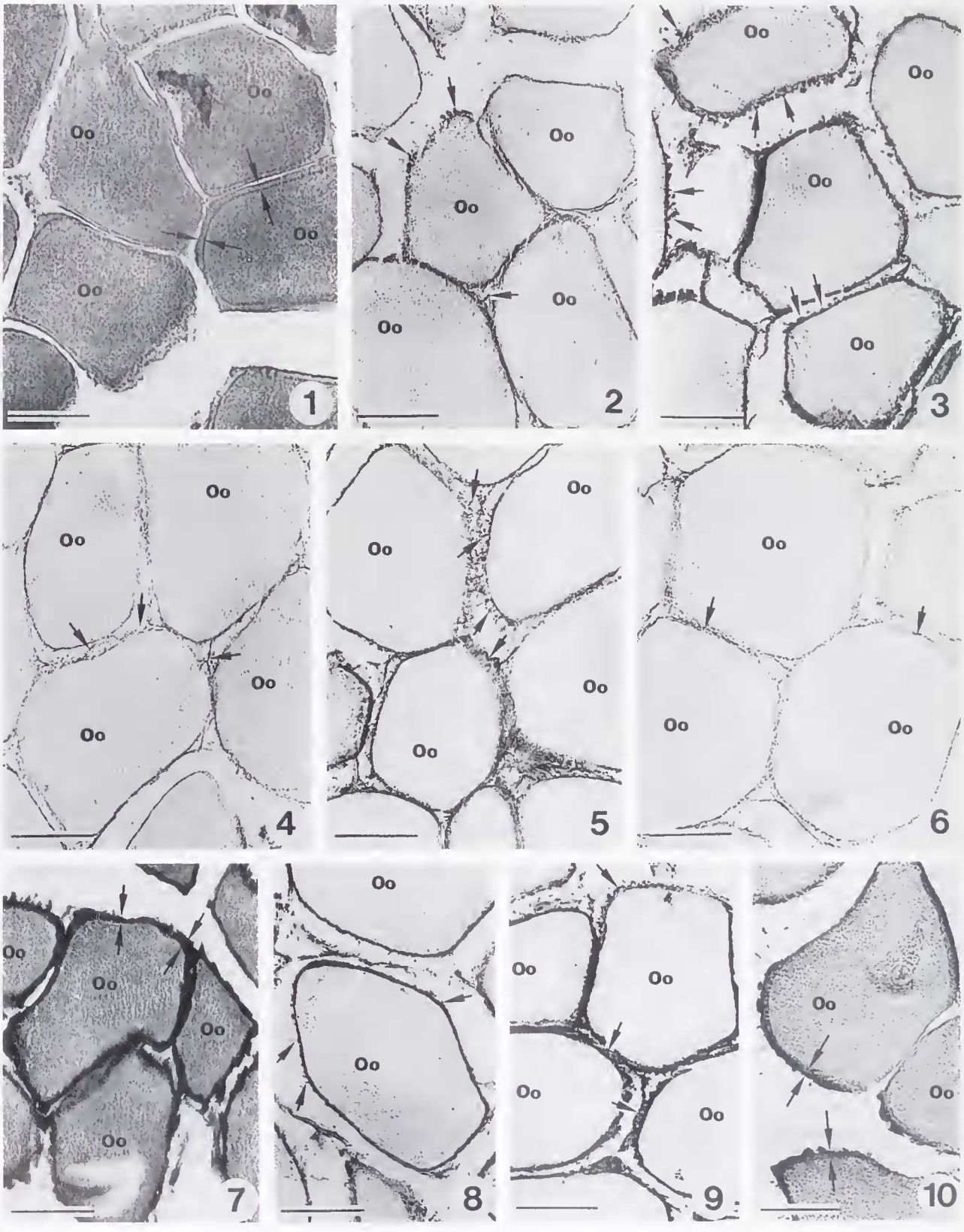
Several specimens of *M. glacialis* were collected in the intertidal zone of the North Atlantic, 30 km north of Oporto, Portugal. The animals were maintained in the laboratory in well-aerated seawater.

For histochemical studies of the jelly coat of the oocyte, mature ovaries were fixed in Bouin's fluid for 24 h, processed through alcohol and benzene, and embedded in paraffin (Ganter and Jollès, 1969). Sections were cut at 7 μm. The following histochemical methods were employed, all without counterstaining:

(1) Alcian blue (1%) 8GX (Sigma) staining at pH 0.5 (Ab pH 0.5) according to the method of Lev and Spicer

Received 27 August 1992; accepted 6 July 1993.

* Address correspondence to this author at the Laboratory of Cell Biology, Institute of Biomedical Sciences, University of Oporto, Lg. Prof. Abel Salazar 2, 4000 Porto, Portugal.



Figures 1–10. Histochemical staining of the jelly coat (JC) of mature oocytes (Oo) in the ovary of *Marthasterias glacialis*. Bar = 50 μm.

to demonstrate sulfate groups, and at pH 2.5 (Ab pH 2.5) according to the method of Wagner and Shapiro to demonstrate those acid groups which remain ionized at and above this pH (Lev and Spicer, 1964; Ganter and Jollès, 1969, 1970).

(2) Methylation to modify functional groups (carboxyl and sulfate) of polysaccharides (Ganter and Jollès, 1969, 1970). Sections were collodionized and then immersed in a solution consisting of liquid HCl diluted to 1% with liquid absolute methanol, at 37°C (mild methylation) or at 60°C (active methylation) for 4–12 h. Some of the sections were then saponified to reverse the methylation of carboxylic acids (1% KOH in 70% ethanol at room temperature for 30 min). After methylation or saponification, sections were stained with Ab pH 0.5 and Ab pH 2.5. Methylation was also performed as in Pearse (1968), with 0.1 *N* HCl-absolute methanol for 8–96 h at 37°C (mild) or at 60°C (drastic).

(3) Alcian blue/critical-electrolyte-concentration technique (Ab-CEC) to identify the different negatively charged acidic groups (Scott and Dorling, 1965; Pearse, 1968; Scott, 1985), by using different $MgCl_2$ concentrations in 3% acetic acid, 0.1% Ab.

(4) The standard periodic acid-Schiff (PAS) reaction for vicinal hydroxyl groups of polysaccharides according to McManus (Pearse, 1968; Ganter and Jollès, 1969, 1970).

(5) Aldehyde fuchsin (AF) staining according to Halmi and Davies (Pearse, 1968; Ganter and Jollès, 1969, 1970) to stain carboxyl and sulfated groups by using basic fuchsin and paraldehyde.

(6) The sequential staining procedures of Ab pH 0.5-PAS and Ab pH 2.5-PAS to differentiate neutral and acidic polysaccharides, and AF-Ab pH 2.5 to distinguish sulfated from weakly acidic polysaccharides (Spicer, 1965; Pearse, 1968; Ganter and Jollès, 1969, 1970).

(7) Mercury-bromophenol blue (Hg-BPB) staining to detect total protein (Pearse, 1968; Ganter and Jollès, 1969, 1970).

The oocyte JC was isolated by the acid method of Matsui *et al.* (1986a). Mature female gonads were cut in Millipore-filtered (0.2 μm) seawater (FSW) at pH 8, and the liberated oocytes were left in FSW until their spontaneous maturation, which is characterized by loss of follicular cells and breakdown of germinal vesicles (Meijer *et al.*, 1984). JC was obtained by washing the oocytes with 20 vol of FSW, resuspending them in 3 vol of FSW, and then lowering the pH to 5.0 with 0.1 *N* HCl. Dejellied oocytes were removed by centrifugation for 5 min at 1500 rpm, and the jelly supernatant was recentrifuged for 30 min at 10,000 rpm at 4°C. The resulting supernatant was adjusted to pH 8.0 with 0.1 *N* NaOH, dialysed against distilled water (Spectra/Por, MW cutoff ×3500) and then lyophilized (SeGall and Lennarz, 1979). Protein concentrations were determined by the method of Lowry *et al.* (1951).

Glycosaminoglycans (GAGs) were isolated from JC according to Whiteman (1972, 1973a,b) as modified by Whiteman and Henderson (1977). To 3 ml of jelly solution (4 mg/ml water), 30 ml of an Ab solution (5 ml of 1% aqueous Ab, 2.65 ml of 2 M $MgCl_2$, 10 ml of 0.5 *M* Na-acetate pH 5.8, and 82.35 ml water) was added. The GAG-Ab complex was then left to equilibrate for 2 h at room temperature (rt). After centrifuging for 15 min at 5000 rpm, 200 μl of 4 *M* NaCl and 100 μl of methanol were added to the pellet with vortex mixing. Then, 100 μl of 0.1 *M* Na_2CO_3 and 400 μl of water were added with vortex mixing. The solution was left to equilibrate for 30 min at rt, and then recentrifuged as above. To 600 μl of the clear supernatant, 1.8 ml of ethanol was added. The solution was left for 10 min at rt, and finally centrifuged as above. The precipitate was left to dessicate overnight and then was resuspended in 50 μl of water. After cen-

Figure 1. Hemalumen-eosin. The JC is the layer that appears uniformly stained (between arrows) all around the oocytes (Oo).

Figures 2 and 3. Alcian blue (Ab) at pH 2.5 to demonstrate the acidic groups that remain ionized at and above this pH, and at pH 0.5 to demonstrate sulfate groups. The JC appears discontinuously stained, giving a mesh-like or fibrillar appearance (arrows). The staining is more intense at pH 0.5 (Fig. 3).

Figures 4–6. Ab/critical-electrolyte-concentration technique at 0.0 *M*, 0.2 *M*, and 0.6 *M* $MgCl_2$ to identify the different negatively charged acidic groups. The JC is discontinuously stained (arrows), revealing a mesh-like or fibrillar component. The staining increases until 0.2 *M* (Fig. 5) and then decreases, becoming almost negative after 0.6 *M* $MgCl_2$ (Fig. 6).

Figure 7. The standard periodic acid-Schiff reaction for vicinal hydroxyl groups of polysaccharides. The JC is intensely and uniformly stained (between arrows).

Figures 8 and 9. Aldehyde-fuchsin (AF) to stain carboxyl and sulfated groups, and AF-Ab pH 2.5 to distinguish sulfated from weakly acidic polysaccharides. The JC is discontinuously stained, giving a mesh-like or fibrillar appearance (arrows). Much more fibrillar material appears stained after AF-Ab pH 2.5 (Fig. 9).

Figure 10. Mercury-bromophenol blue reaction for total protein. The entire JC appears stained (between arrows).

Figure 11. Cellulose acetate electrophoresis of total jelly coat. Alcian blue staining (a). Three bands can be observed, of which one remained at the origin (O) and two migrated toward the anode with intermediary (**) and faster (*) mobilities. Alcian blue/critical-electrolyte-concentration technique (Ab-CEC) at different concentrations of $MgCl_2$: (b) 0.1 *M*, (c) 0.2 *M*, (d) 0.4 *M*, (e) 0.6 *M*, (f) 0.8 *M*, (g) 1 *M*, and (h) 1.5 *M*. The critical electrolyte concentration of the intermediary moving band (**) is 0.4 *M* (d) and that of the faster moving band (*) is 0.6 *M* (e), whereas the staining of the band at the origin (O) shows an intense decrement after 0.6 *M* $MgCl_2$ (f). Only the band at the origin is stained by coomassie blue (i) and standard periodic acid-Schiff (PAS) reaction (j).

trifuging as above, the supernatant was used for electrophoresis.

Concentration of GAGs was determined by the method of Whiteman (1972, 1973a,b). To 25 and 50 μl of a 1-mg jelly/ml water solution (in triplicate), 1 ml of the above Ab solution was added. After 4 h at rt, the solution was centrifuged as above, and then 2 ml of ethanol was added to the pellet with vortex mixing. After centrifuging as above, the precipitate was dissociated with 1 ml of 7.5% aqueous SDS by vortex mixing. After 30 min at rt, solutions were read at 678 nm in a Pye-Unicam SP6-550 spectrophotometer. The standard calibration curve was run with a starting solution of chondroitin-4-sulfate, 200 μg/ml water, over the range of 0–10 μg/ml.

For monodimensional cellulose acetate electrophoresis (CAE), Cellogel sheets, 5.7 × 14 cm, were previously equilibrated in electrophoresis buffer (0.05 *M* calcium acetate, pH 7.2). Samples (10–20 μl/lane, from a 4-mg jelly/ml aqueous solution or from the GAG precipitate) were applied with an LRE applicator (Medizin Technik). Standards were from Sigma and consisted of 1 mg/ml water of keratan sulfate, dermatan sulfate, heparan sulfate, chondroitan sulfate, hyaluronic acid, and heparin. Electrophoresis was performed in an electrophoresis apparatus (EP-166 Medizin Technik) at a constant voltage of 200 V for 1 h (14 V/cm). For bidimensional CAE, Cellogel sheets, 14 × 14 cm, were equilibrated in 0.1 *M* pyridine, 0.47 *M* formic acid, pH 3, and the first run was for 1 h at 100 V. For the second dimension, electrophoresis was performed in 0.05 *M* calcium acetate, pH 7.2, for 2 h at 200 V (Hata and Nagai, 1972). Sheets were then stained with coomassie blue R (Fluka AG) (Cb), PAS, or Ab-CEC. For Ab-CEC (Scott, 1985; Wall and Gyi, 1988), gels were stained overnight and then washed and stored in the same CEC solution without Ab. For general Ab staining, a 0.5% solution in 7% acetic acid was used. For the PAS reaction, acetic acid fixed gels were washed in 3% acetic acid for 15 min, oxidized for 1 h at 4°C with fresh 1% periodic acid in 3% acetic acid, washed three times (5 min each) with water, and stained overnight at 4°C in the Merck Schiff reagent.

For enzymatic treatments, JC or the GAG precipitate was submitted to the following digestions: chondroitinase ABC (Sigma, from *Proteus vulgaris*, 0.55 U/mg) was used at 0.25 and 0.75 U/mg JC for 3 and 24 h at 37°C in 0.1 *M* Tris-HCl–0.1 *M* Na-acetate, pH 7.3 (Yanagishita *et al.*,

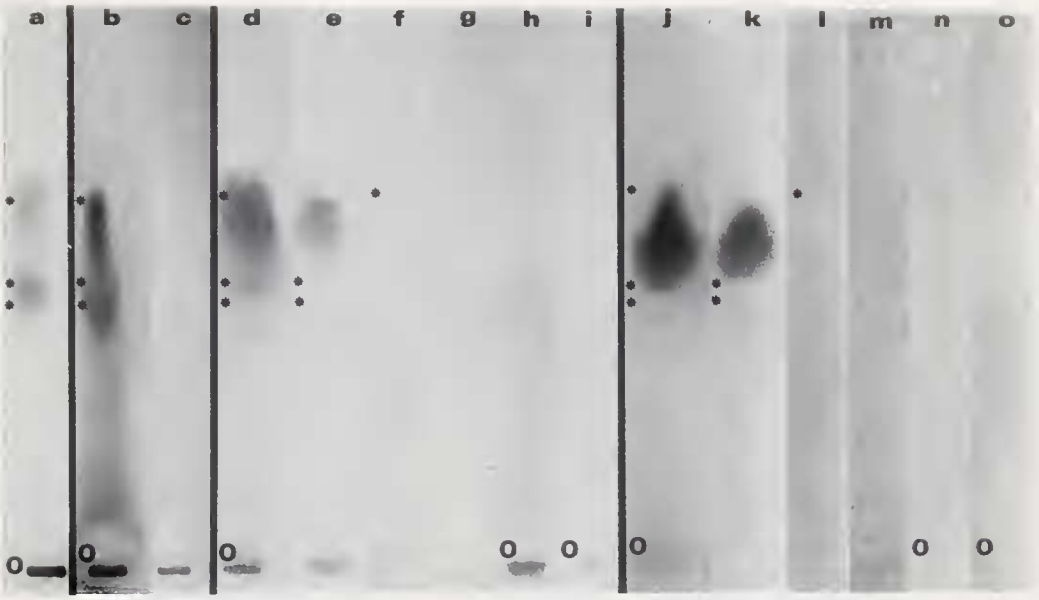


Figure 12. Cellulose acetate electrophoresis of total jelly coat after enzymatic digestions. Chrondroitinase ABC digestion: Alcian blue staining (a). No changes are observed on the band at the origin (O), or on the intermediary (**) and faster (*) moving bands. Neuraminidase digestion: Alcian blue (b) and standard periodic acid-Schiff (PAS) reaction (c). The intermediary (**) and faster (*) moving bands appear partially digested, producing a smear in which both bands are still visible (b), with no changes apparently having occurred on the band at the origin [O] (b) and (c). Hyaluronidase digestion: (d) through (i). The alcian blue (d) and PAS (i) staining of the band at the origin (O) is decreased, but no changes are noticed (h) with coomassie blue. The intermediary (**) and faster (*) moving bands also seem to be partially digested—their independent configurations changed to a large smear (d). A minimal amount of this smear presents a CEC coincident with that of the intermediary moving band (**) [(e), 0.4 *M* $MgCl_2$], whereas most of the smear shows a CEC coincident with that of the faster moving band (*) [(f), 0.6 *M* $MgCl_2$]. Control strip containing only the enzyme (g). Protease digestion: (j) through (o). A large smear is observed at the level of the intermediary (**) and faster (*) moving bands with alcian blue staining (j). The CEC of this smear is coincident with that of the faster moving band (*) [(l), 0.6 *M* $MgCl_2$] and not with that of the intermediary moving band (**) [(k), 0.4 *M* $MgCl_2$]. The band at the origin (O) appears totally digested—no staining is noticed after alcian blue (j), coomassie blue (n), or PAS (o) staining. Control strip containing only the enzyme (m).

1979; Heinegard and Sommarin, 1987); hyaluronidase (Sigma, from sheep testes, type V, 2000 U/mg) was used at 50, 100, and 200 U/mg JC for 3 and 24 h at 37°C in 0.15 *M* NaCl, 0.1 *M* Na-acetate, 0.001 *M* disodium EDTA, pH 5 (Cowman *et al.*, 1981; Turner and Cowman, 1985; Min and Cowman, 1986); neuraminidase (Sigma, type V, from *Clostridium perfringens*, 1.7 U/mg) was used at 25 and 100 U/mg JC for 24 h at 37°C in 0.1 *M* Na-acetate pH 5.3 with 0.04 *M* $CaCl_2$ (Kudo, 1982; Delgado and Zoller, 1987); trypsin (Sigma, type III, from bovine pancreas, 11,680 U/mg) was used at 10 μg and 0.3 mg/mg JC, protease (Sigma, type VI, from *Streptomyces griseus*, 5 U/mg) and pronase E (Merck, from *Streptomyces griseus*, 95,000 PUK/g) were used at 2 mg/mg JC, all three for 3 and 24 h at 37°C in 0.1 *M* Tris-HCl, 0.1 *M* Na-acetate, pH 7.3 (Yanagishita *et al.*, 1979; Grimek and Ax, 1982; Heinegard and Sommarin, 1987; Huey *et al.*, 1990). Digestions were terminated by boiling the samples for 5–10 min (Cowman *et al.*, 1981). Samples were then applied to CAE as above.

Results

Light microscopy

Ab discontinuously stained the JC of mature *M. glacialis* oocytes. The reaction was more intense at pH 0.5 than at 2.5 (Figs. 1–3), thus demonstrating on this mesh-like or fibrillar component the presence of both sulfated and carboxylic groups, with the predominance of the former (Lev and Spicer, 1964; Ganter and Jollès, 1969, 1970). Methylation abolished staining with Ab, but sections that were mildly methylated could be partly restained with Ab at both pHs after saponification with KOH. Methylation, which blocks carboxylic and eliminates sulfated groups (Ganter and Jollès, 1969, 1970), did not inhibit Ab pH 0.5 staining after saponification, indicating that this staining is not specific for sulfated groups alone and that the stronger reaction obtained at that pH cannot be assumed to be due to a sulfate-rich polysaccharide. In the presence of $MgCl_2$ (Ab-CEC), the intensity of Ab staining gradually increased until 0.2 *M* and then de-

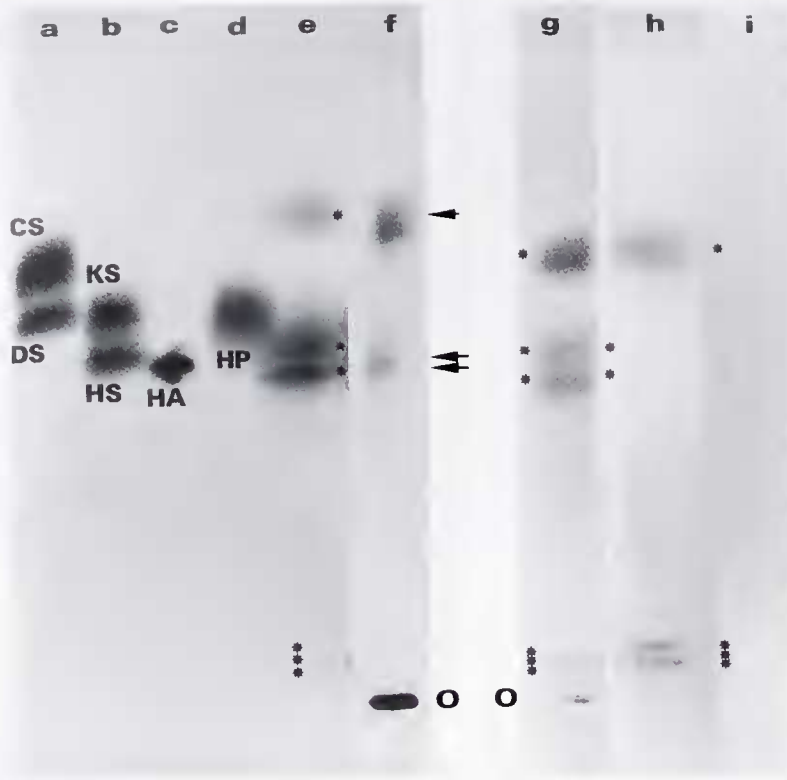


Figure 13. Cellulose acetate electrophoresis of GAG-standards [(a) through (d)] and GAG-like molecules [(e) through (i)] after staining with alcian blue [(a) through (f)] and alcian blue/critical-electrolyte-concentration technique (Ab-CEC) at different concentrations of $MgCl_2$ [(g), 0.2 *M*, (h), 0.4 *M*, (i), 0.6 *M* $MgCl_2$]. Standards are (a) chondroitan sulfate (CS) and dermatan sulfate (DS), (b) keratan sulfate (KS) and heparan sulfate (HS), (c) hyaluronic acid (HA), and (d) heparin (HP). Molecules in (e), (g), (h), and (i) were isolated from total jelly coat; molecules in (f) were those of the total jelly coat. Isolated GAG-like molecules separated into three moving bands (e). Two of these bands (**) had an R_f similar to that of the intermediary moving band of total jelly coat [(f), double arrow], and one (*) had an R_f similar to that of the faster moving band of total jelly coat [(f), arrow]. A minor component (***) can also be seen near or at the origin (O) [(e), (g), (h)]. The intermediary moving bands (**) of isolated GAG-like molecules seem to comigrate with HS and HA, whereas the faster moving band (*) migrates slightly above CS (e). The CEC of the intermediary moving bands (**) is 0.4 *M* $MgCl_2$ (h) and that of the faster moving band (*) and the minor bands (***) near the origin (O) is 0.6 *M* $MgCl_2$ (i).

ereased to become almost negative after 0.6 *M* (Figs. 4–6). These results suggest that the Ab-stained material is not highly sulfated and that earboxylie groups predominate, because strong staining was found only in the presenee of low $MgCl_2$ eoncentrations (Seott and Dorling, 1965; Scott, 1985).

Contrary to Ab staining, PAS and Ab pH 2.5-PAS uniformly stained the whole jelly layer an intense pink and purple, respectively (Fig. 7), thus revealing the presenee of a seeond maeromolecular eomponent made up of neutral or weakly aeidie polysaccharides (Pearse, 1968; Ganter and Jollès, 1969, 1970; Rambourg, 1971).

When seetions were stained with AF, the fibrillar eomponent of the JC stained purple but, after the sequence AF-Ab pH 2.5, mueh more fibrillar material appeared stained with intense purple and blue (Figs. 8, 9), whieh suggests the presenee of both carboxylie and sulfated groups in the fibrillar component of the JC, although with a predominance of the former (Pearse, 1968; Ganter and Jollès, 1969, 1970).

With protein staining, the Hg-BPB reaction gave a strong blue stain to the entire JC (Fig. 10), suggesting that JC polysaeeharides may be associated with proteins.

Cellulose acetate electrophoresis

When JC was submitted to CAE and then stained with Ab, three bands appeared, of which one remained at the origin and two migrated toward the anode (Fig. 11a). With the Ab-CEC staining teehnique, the bands of intermediary and faster mobilities showed a eritical eleetrolytieal point at 0.4 and 0.6 *M* $MgCl_2$, respectively, whereas the staining

Figure 14. Bidimensional cellulose acetate electrophoresis of GAG-like molecules isolated from total jelly coat (alcian blue staining). Standards of first dimension are hyaluronic acid (HA), keratan sulfate (KS), and dermatan sulfate (DS). In this buffer system (pH 3), heparan sulfate (HS) comigrates with KS. Standards of second dimension are HS, KS, and chondroitan sulfate (CS). In this buffer system (pH 7.2), HA comigrates with HS, and DS with KS. Isolated GAG-like molecules separated into three bands, of which two (**) seem to migrate near to HS and HA and then may correspond to the intermediary moving bands (Fig. 13), and one (*) that migrates near CS and then may correspond to the faster moving band (Fig. 13).

of the band at the origin showed an intense decrement after 0.6 *M* $MgCl_2$ (Fig. 11b–h). Only the band that remained at the origin was stained by Cb and PAS (Fig. 11i, j). Protein determination gave a value of 190 μg protein/mg JC, or about 19% of the total JC. These biochemical findings, together with the histochemical results obtained with PAS and Ab pH 2.5-PAS stainings, suggest that the neutral or weakly acidic JC component, as detected on tissue sections, is mainly contained in the band that remained at the origin.

The Ab-CEC staining of tissue sections, which gave a maximum staining of the JC fibrillar material at 0.2 *M* and an almost absence of staining after 0.6 *M* $MgCl_2$, further suggest that this JC component may have mainly separated after CAE into the two moving bands. All three bands appeared resistant to chondroitinase ABC digestion (Fig. 12a). Neuraminidase seemed to digest the moving band slightly, producing a smear in which both bands are still visible (Fig. 12b, c). After hyaluronidase treatment, Ab and PAS staining of the band at the origin appeared decreased, but no change was noticed with Cb staining. This suggests the presence of both polysaccharides and proteins on that band, with polysaccharides being partially digested by the enzyme (Fig. 12d–i). The two moving bands seemed also to have been partially attacked by hyaluronidase, because their independent configurations changed to a large smear (Fig. 12d). Most of the smear, however, showed a critical electrolyte point coincident with that of the faster moving band, thus suggesting that the intermediary moving band was mainly digested by the enzyme (Fig. 12e, f). Protease seemed to have mainly digested the intermediary moving band, because the moving residual band presents a critical electrolyte point coincident with that of the faster moving band (Fig. 12j–l). However, the configuration of the faster moving band also changed, producing a large smear band, which suggests that it has been partially attacked by the enzyme (Fig. 12j–l). The band at the origin appeared totally digested by protease, as no staining was noticed after Ab, Cb, and PAS stainings (Fig. 12j–o). This indicates that proteins on this band may be structurally linked to polysaccharides, because digestion of the band enabled polysaccharides to move and escape detection.

Glycosaminoglycan-like materials were isolated from the JC and shown to constitute about 20% of the total JC. After CAE, they separated into three main moving bands, two that present an R_f similar to that of the intermediary moving band of total JC and one whose R_f is similar to that of the faster moving band of total JC (Fig. 13). A minor component was also observed near the origin (Fig. 13). This latter finding suggests that the intense Ab staining of the band at the origin of total JC is due not to the presence of a large GAG-like component but to the staining of the weakly acidic groups of the JC PAS-positive component. The presence of GAG-like materials in the same positions as on the main moving bands of total JC was further confirmed by CEC experiments. These experiments showed coincident critical electrolytical points, thus suggesting that the GAG-like materials were extracted from the moving bands (Fig. 13).

In relation to standards, the two intermediary moving bands seemed to comigrate with heparan sulfate and hyaluronic acid, while the faster moving band migrated slightly above chondroitan sulfate (Fig. 13). Similar relationships to standards could also be established after bidimensional CAE (Fig. 14). Chondroitinase ABC mainly attacked the faster moving band, whereas protease and hyaluronidase seemed to have digested all bands (Fig. 15). These results thus suggest that the Ab-precipitated materials from total JC may be proteoglycan-like, containing

GAG-like materials similar to chondroitan sulfate in the faster moving band and heparan sulfate/hyaluronic acid in the intermediary moving bands. The reason that chondroitinase ABC attacked the intermediary moving band and hyaluronidase digested all bands is that they can act in hyaluronate and chondroitan sulfate, respectively (Heinegard and Sommarin, 1987). The lack of a clear R_f relationship between isolated GAGs and standards may be due to the presence of associated proteins or may indicate that they are only partially similar in structure. The different results obtained with enzymatic digestions of total JC and isolated GAG-like materials may be explained by the different accessibility of enzymes to GAG-like molecules in total JC.

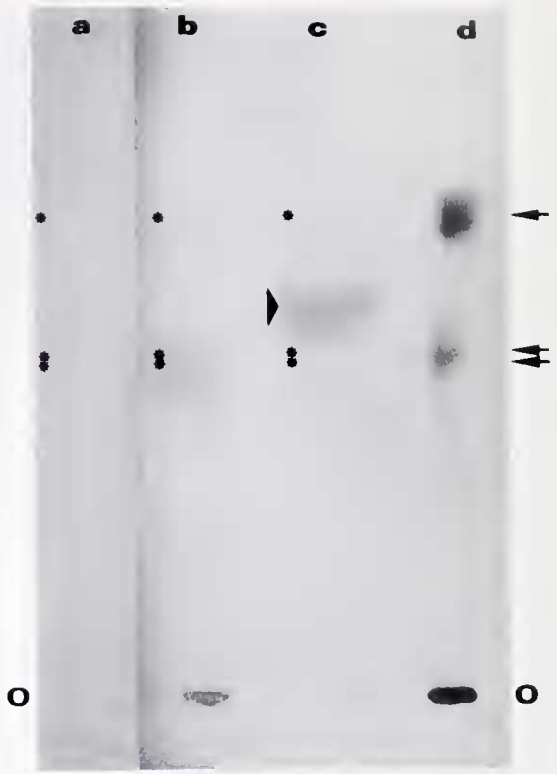


Figure 15. Cellulose acetate electrophoresis of GAG-like molecules isolated from total jelly coat after enzymatic digestions (alcian blue staining). (a) Hyaluronidase seems to have digested all bands, because no band appears stained. (b) Chondroitinase ABC seems to have partially digested the band at the origin (O) (because its staining appears decreased) and the intermediary moving band (**), producing a large smear. This enzyme seems to have totally digested the faster moving band (*), as no band is noticed at that level. (c) Proteinase seems to have attacked all bands, since no band appears stained. The band (arrowhead) that appears between the intermediary (**) and faster (*) moving bands may represent residual GAG-like materials liberated from the digested bands. (d) Total jelly coat under the same conditions but without enzymatic treatment (O, band at the origin; double arrow, intermediary moving band; arrow, faster moving band).

Discussion

The histochemical study of the JC of *M. glacialis* oocytes revealed the presence of two major types of polysaccharides: an acidic fibrillar material, which seems to contain carboxylic (uronic/sialic) and sulfated groups; and a loose component, which seems to contain neutral or weakly acidic polysaccharides with vic-glycols near carboxylic groups. On the basis of protein staining, these structures may be associated with proteins. As with the starfish, the JC of sea urchin oocytes shows the presence of neutral and acidic polysaccharides that contain carboxylic and sulfated groups in proximity to vicinal hydroxyls, but the JC appears much more sulfated, and its acidic component could not be morphologically distinguished from the neutral component (Jondeung and Czihak, 1982).

SeGall and Lennarz (1979) suggested that the polysaccharide component of the JC of sea urchins is made up of a sialoprotein and a fucose sulfate glycoconjugate. In addition to these two components, the JC was shown to contain small peptides (SAP) that are chemoattractant (*e.g.*, resact) and motility activating for sperm (Garbers, 1989; Suzuki, 1990; Hoshino *et al.*, 1992; Harumi *et al.*, 1992; Suzuki and Yoshino, 1992; Yoshino and Suzuki, 1992; Yoshino *et al.*, 1992). Recently, the fucose sulfate glycoconjugate was shown to contain several proteins that, using SAP-I as cofactor, induce the acrosomal reaction of the sperm, and a fucose sulfate polymer that is responsible for sperm agglutination (Garbers *et al.*, 1983; Yamaguchi *et al.*, 1989; Shimizu *et al.*, 1990; Mikami-Takei *et al.*, 1991).

In the starfish, the JC was demonstrated to contain a large sulfated glycoprotein (ARIS) and a group of steroidal saponins (Co-ARIS), which induce the acrosomal reaction and the degradation of sperm histones; an oligopeptide (SAP) that participates in the induction of the acrosomal reaction and stimulates sperm respiration; and a high-mannose glycoprotein (Uno and Hoshi, 1978; Ikadai and Hoshi, 1981a, 1981b; Matsui *et al.*, 1986a, 1986b; Endo *et al.*, 1987; Nishiyama *et al.*, 1987a, 1987b; Hoshi *et al.*, 1990a, 1990b; Amano *et al.*, 1992).

Although GAGs are known components of the cumulus of mammalian oocytes, they have not yet been reported in the jelly layer of echinoderm oocytes. Alcian blue, which is known to selectively precipitate GAGs (Reale *et al.*, 1986), specifically stained the outer region and some fibrillar extensions of the JC of *M. glacialis* oocytes (Sousa and Azevedo, 1988) as well as some high molecular weight components extracted from it (Sousa *et al.*, 1992). Based on these results and on the present histochemical experiments on ovary sections, GAGs were isolated from the JC of *M. glacialis* oocytes and qualitatively analyzed by cellulose acetate electrophoresis under different conditions. Taking into consideration the relative migration

rate of these materials and of standard GAGs, as well as their sensitivities to chondroitinase ABC and hyaluronidase digestions, it is possible to suggest that the JC may contain GAG-like molecules that bear some resemblance to chondroitan sulfate, heparan sulfate, and hyaluronic acid. Quantitative experiments are being performed to confirm these findings.

Acknowledgments

This work was partially supported by CME-INIC and Fund. Eng. A. Almeida. We thank Mr. Vítor Oliveira for technical help and Mr. João Carvalheiro for the iconographic work.

Literature Cited

Amano, T., Y. Okita, and M. Hoshi. 1992. Treatment of starfish sperm with egg jelly induces the degradation of histones. *Dev. Growth Differ.* **34:** 99–106.

Cowman, M. K., E. A. Balazs, C. W. Bergmann, and K. Meyer. 1981. Preparation and circular dichroism analysis of sodium hyaluronate oligosaccharides and chondroitin. *Biochemistry* **20:** 1379–1385.

Delgado, M. V., and L. C. Zoller. 1987. A quantitative and qualitative cytochemical analysis of glycosaminoglycan content in the zona pellucida of hamster ovarian follicles. *Histochemistry* **87:** 279–287.

Endo, T., M. Hoshi, S. Endo, Y. Arata, and A. Kobata. 1987. Structures of the sugar chains of a major glycoprotein present in the egg jelly coat of a starfish, *Asterias amurensis*. *Arch. Biochem. Biophys.* **252:** 105–112.

Ganter, P., and G. Jollès. 1969. Histochimie Normale et Pathologique. Vol. 1. Gauthier-Villars, Paris.

Ganter, P., and G. Jollès. 1970. Histochimie Normale et Pathologique. Vol. 2. Gauthier-Villars, Paris.

Garbers, D. L. 1989. Molecular basis of signalling in the spermatozoon. *J. Androl.* **10:** 99–107.

Garbers, D. L., G. S. Kopf, D. J. Tubb, and G. Olson. 1983. Elevation of sperm 3',5'-monophosphate concentrations by a fucose-sulfate-rich complex associated with eggs: I. structural characterization. *Biol. Reprod.* **29:** 1211–1220.

Grimek, H. J., and R. L. Ax. 1982. Chromatographic comparison of chondroitin-containing proteoglycan from small and large bovine ovarian follicles. *Biochem. Biophys. Res. Commun.* **104:** 1401–1406.

Harumi, T., K. Hoshino, and N. Suzuki. 1992. Effects of sperm-activating peptide I on *Hemicentrotus pulcherrimus* spermatozoa in high potassium sea water. *Dev. Growth Differ.* **34:** 163–172.

Hata, R., and Y. Nagai. 1972. A rapid and micro method for separation of acidic glycosaminoglycans by two-dimensional electrophoresis. *Anal. Biochem.* **45:** 462–468.

Heinegard, D., and Y. Sommarin. 1987. Isolation and characterization of proteoglycans. *Methods Enzymol.* **144:** 319–372.

Hoshi, M., T. Amano, Y. Okita, T. Okinaga, and T. Matsui. 1990a. Egg signals for triggering the acrosome reaction in starfish spermatozoa. *J. Reprod. Fert. Suppl.* **42:** 23–31.

Hoshi, M., T. Amano, Y. Okita, T. Okinaga, and T. Matsui. 1990b. Induction of the acrosome reaction in starfish. Pp. 239–252 in *Mechanism of Fertilization: Plants to Humans*, B. Dale, ed. NATO ASI Series, Series H: Cell Biology, Vol. 45, Springer-Verlag, Berlin.

Hoshino, K., T. Shimizu, Y. Sendai, T. Harumi, and N. Suzuki. 1992. Differential effects of the egg jelly molecules FSG and SAP-I on elevation of intracellular Ca^{2+} and pH in sea urchin spermatozoa. *Dev. Growth Differ.* **34:** 403–411.

Huey, G., A. Moiin, and R. Stern. 1990. Levels of [^{3}H]glucosamine incorporation into hyaluronic acid by fibroblasts is modulated by culture conditions. *Matrix* **10:** 75–83.

Ikadai, H., and M. Hoshi. 1981a. Biochemical studies on the acrosome reaction of the starfish, *Asterias amurensis*. I. Factors participating in the acrosome reaction. *Dev. Growth Differ.* **23:** 73–80.

Ikadai, H., and M. Hoshi. 1981b. Biochemical studies on the acrosome reaction of the starfish, *Asterias amurensis*. II. Purification and characterization of acrosome reaction-inducing substance. *Dev. Growth Differ.* **23:** 81–88.

Jondeung, A., and G. Czihak. 1982. Histochemical studies of jelly coat of sea-urchin eggs during oogenesis. *Histochemistry* **76:** 123–136.

Kudo, S. 1982. Ultrastructure and ultracytochemistry of fertilization envelope formation in the carp egg. *Dev. Growth Differ.* **24:** 327–339.

Lev, R., and S. S. Spicer. 1964. Specific staining of sulphate groups with alcian blue at low pH. *J. Histochem. Cytochem.* **12:** 309.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin reagent. *J. Biol. Chem.* **193:** 265–275.

Matsui, T., I. Nishiyama, A. Hino, and M. Hoshi. 1986a. Induction of the acrosome reaction in starfish. *Dev. Growth Differ.* **28:** 339–348.

Matsui, T., I. Nishiyama, A. Hino, and M. Hoshi. 1986b. Acrosome reaction-inducing substance purified from the egg jelly inhibits the jelly-induced acrosome reaction in starfish: an apparent contradiction. *Dev. Growth Differ.* **28:** 349–357.

Meijer, L., P. Pondaven, P. Guerrier, and M. Moreau. 1984. A starfish oocyte user's guide. *Cah. Biol. Mar.* **45:** 457–480.

Mikami-Takei, K., M. Kosakai, M. Isemura, T. Suyemitsu, K. Ishihara, and K. Schmid. 1991. Fractionation of jelly substance of the sea urchin egg and biological activities to induce acrosome reaction and agglutination of spermatozoa. *Exp. Cell Res.* **192:** 82–86.

Miller, D. J., and R. L. Ax. 1990. Carbohydrates and fertilization in animals. *Mol. Reprod. Dev.* **26:** 184–198.

Min, H., and M. K. Cowman. 1986. Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution. *Anal. Biochem.* **155:** 275–285.

Nishiyama, I., T. Matsui, and M. Hoshi. 1987a. Purification of Co-ARIS, a cofactor for acrosome-inducing substance, from the egg jelly of starfish. *Dev. Growth Differ.* **29:** 161–169.

Nishiyama, I., T. Matsui, Y. Fujimoto, N. Ikekawa, and M. Hoshi. 1987b. Correlation between the molecular structure and the biological activity of Co-ARIS, a cofactor for acrosome reaction-inducing substance. *Dev. Growth Differ.* **29:** 171–176.

Pearse, A. G. E. 1968. *Histochemistry, Theoretical and Applied.* Vols. 1 and 2, J. A. Churchill, London.

Rambourg, A. 1971. Morphological and histochemical aspects of glycoproteins at the surface of animal cells. *Int. Rev. Cytol.* **31:** 57–114.

Reale, E., L. Luciano, and M. Spitznas. 1986. Histochemical demonstration of hyaluronic acid molecules by alcian blue. *Histochem. J.* **18:** 306–316.

Scott, J. E. 1985. Proteoglycan histochemistry—a valuable tool for connective tissue biochemists. *Collagen Res. Rel.* **5:** 541–575.

Scott, J. E., and J. Dorling. 1965. Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. *Histochemie* **5:** 221–233.

SeGall, G. K., and W. J. Lennarz. 1979. Chemical characterization of the component of the jelly coat from sea urchin eggs responsible for induction of the acrosome reaction. *Dev. Biol.* **71:** 33–48.

Shimizu, T., H. Kinoh, M. Yamaguchi, and N. Suzuki. 1990. Purification and characterization of the egg jelly macromolecules, sialo-

glycoprotein and fucose sulfate glycoconjugate, of the sea urchin *Hemicentrotus pulcherrimus*. *Dev. Growth Differ.* **32:** 473–487.

Sousa, M., and C. Azevedo. 1988. Ultrastructural and histochemical observations of the cortical reaction in *Marthasterias glacialis* (Echinodermata, Asteroidea). *J. Submicrosc. Cytol. Pathol.* **20:** 629–633.

Sousa, M., P. Moradas-Ferreira, and C. Azevedo. 1992. Presence of a trypsin-like protease in starfish sperm acrosome. *J. Exp. Zool.* **261:** 349–354.

Spicer, S. S. 1965. Diamine methods for differentiating mucosubstances histochemically. *J. Histochem. Cytochem.* **13:** 211–234.

Suzuki, N. 1990. Structure and function of egg-associated peptides of sea urchins. Pp. 271–286 in *Mechanism of Fertilization: Plants to Humans*, B. Dale, ed., NATO ASI Series, Series H: Cell Biology, Vol. 45, Springer-Verlag, Berlin.

Suzuki, N., and K.-I. Yoshino. 1992. The relationship between amino acid sequences of sperm-activating peptides and the taxonomy of echinoids. *Comp. Biochem. Physiol.* **102B:** 679–690.

Turner, R. E., and M. K. Cowman. 1985. Cationic dye binding by hyaluronate fragments: dependence on hyaluronate chain length. *Arch. Biochem. Biophys.* **237:** 253–260.

Uno, Y., and M. Hoshi. 1978. Separation of the sperm agglutinin and the acrosome reaction-inducing substance in egg jelly of starfish. *Science* **200:** 58–59.

Wall, R. S., and T. J. Gyi. 1988. Alcian blue staining of proteoglycans in polyacrylamide gels using the critical electrolyte concentration approach. *Anal. Biochem.* **175:** 298–299.

Whiteman, P. D. 1972. A new method for the determination of acid glycosaminoglycans in urine. *Biochem. J.* **127:** 87–88.

Whiteman, P. 1973a. The quantitative measurement of alcian blue–glycosaminoglycan complexes. *Biochem. J.* **131:** 343–350.

Whiteman, P. 1973b. The quantitative determination of glycosaminoglycans in urine with alcian blue 8GX. *Biochem. J.* **131:** 351–357.

Whiteman, P., and H. Henderson. 1977. A method for the determination of amniotic-fluid glycosaminoglycans and its application to the prenatal diagnosis of Hurler and SanFilippo diseases. *Clin. Chim. Acta* **79:** 99–105.

Yamaguchi, M., M. Kurita, and N. Suzuki. 1989. Induction of the acrosome reaction of *Hemicentrotus pulcherrimus* spermatozoa by the egg jelly molecules, fucose-rich glycoconjugate and sperm-activating peptide I. *Dev. Growth Differ.* **31:** 233–239.

Yanagishita, M., D. Rodbard, and V. C. Hascall. 1979. Isolation and characterization of proteoglycans from porcine ovarian follicular fluid. *J. Biol. Chem.* **254:** 911–920.

Yoshino, K.-I., and N. Suzuki. 1992. Two classes of receptor specific for sperm-activating peptide III in sand-dollar spermatozoa. *Eur. J. Biochem.* **206:** 887–893.

Yoshino, K.-I., T. Takao, Y. Shimonishi, and N. Suzuki. 1992. Sperm-activating peptide type-V (SAP-V), a fifth member of the sperm-activating peptide family, purified from the egg-conditioned media of the heart urchin *Brissus agassizii*. *Comp. Biochem. Physiol.* **102B:** 691–700.